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***In vivo* implantation of tissue engineered human nasal septal neocartilage constructs: a pilot study**

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Abstract

Objective—To determine the *in vivo* biocompatibility of septal neocartilage constructs developed *in vitro* by an alginate intermediate step.

Study Design—Prospective, animal model.

Setting—Research laboratory.

Subjects and Methods—A murine model was used to examine the maturation of neocartilage constructs *in vivo*. Chondrocytes collected from patients undergoing septoplasty were expanded in monolayer and suspended in alginate beads for three-dimensional culture in media containing human serum and growth factors. After *in vitro* incubation for 5 weeks, the constructs were implanted in the dorsum of athymic mice for 30 and 60 days (n=9). After the mice were sacrificed, the constructs were recovered for assessment of their morphological, histochemical, biochemical, and biomechanical properties.

Results—The mice survived and tolerated the implants well. Infection and extrusion were not observed. Neocartilage constructs maintained their general shape and size, and demonstrated cell viability after implantation. The implanted constructs were firm and opaque, sharing closer semblance to native septal tissue relative to the gelatinous, translucent pre-implant constructs. Histochemical staining with hematoxylin and eosin (H&E) revealed that the constructs exhibited distinct morphologies characteristic of native tissue, which were not observed in pre-implant constructs. DNA and type II collagen increased with duration of implantation, whereas type I collagen and glycoaminoglycans (GAG) decreased. Mechanical testing of a 60-day implanted construct demonstrated characteristics similar to native human septal cartilage.

Conclusions—Neocartilage constructs are viable in an *in vivo* murine model. The histologic, biochemical, and biomechanical features of implanted constructs closely resemble native septal tissue when compared to pre-implant constructs.

Keywords

cartilage tissue engineering; human septal cartilage; murine; nude mouse; *in vivo* tissue engineering

Introduction

Nasal septal cartilage is widely used by head and neck reconstructive surgeons to repair cartilaginous defects of the nose. Its superior supportive properties, relative ease of harvest, and minimal donor site morbidity make it preferable over other autologous cartilage donor sources. The elastin content and innate curvilinear shape of auricular cartilage render it inferior for nasal defect repair. The acquisition of costal cartilage is associated with moderate to significant morbidity and its use is associated with an unpredictable degree of absorption and warping. Septal cartilage is favored over synthetic and allogenic implants, but is limited by the finite amount of available tissue and suboptimal geometric structure for reconstruction of larger craniofacial defects.¹⁻⁴

Tissue engineering of autologous septal neocartilage therefore offers a potential alternative. Large quantities of autologous septal cartilage may be generated from a small specimen and then sculpted to the desired size and shape. Cartilage tissue engineering studies, primarily using articular chondrocytes seeded onto scaffolds, have demonstrated improved histologic and mechanical properties after *in vivo* maturation.⁵⁻⁷ Stiffness and failure load increased significantly when fibrinogen/poly-glycolide-poly-lactide (PLGA)-polymer bovine articular chondrocyte constructs were implanted into nude mice for 6 and 12 weeks. Formation of a chondron-like cell matrix with homogenous distribution of chondrocytes was demonstrated upon staining with hematoxylin and eosin (H&E).

Few reports have focused on the *in vivo* implantation of tissue engineered human septal cartilage. The development of tissue engineered nasal septal constructs that possess the biomechanical and biological properties of native tissue has not yet been achieved. The aim of this study was to determine if *in vivo* implantation will foster maturation of human septal neocartilage constructs cultured in human serum (HS) and developed by the alginate recovered chondrocyte (ARC) method.⁸ Histologic, biochemical, and biomechanical properties of neocartilage constructs were evaluated.

Methods

Chondrocyte Isolation and Expansion

The study used remnant human septal specimens removed during routine surgery at the University of California, San Diego Medical Center or San Diego Veterans Affairs Medical Center (prior IRB approval). The cartilage specimens were dissected free of perichondrium and diced into pieces (1 mm³). The fragments were digested as reported previously.¹⁶ Suspensions of digested cartilage were filtered (70 µm), then washed and centrifuged. Cells

were resuspended in cell culture medium (DMEM [low glucose], 2% pooled human AB serum (Gemini Bioproducts, Woodland, CA), 25 µg/mL ascorbate, 0.4 mmol/L l-proline, 2 mmol/L l-glutamine, 0.1 mmol/L nonessential amino acids, 10 mmol/L HEPES buffer, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B). The number of chondrocytes was determined by hemacytometer counting after trypan blue exclusion.

For each patient, isolated chondrocytes were seeded at 5,000 cells per cm² surface area into T-175 flasks. Monolayer cultures were incubated at 37°C with 5% carbon dioxide/air. Culture medium was supplemented with 1ng/mL transforming growth factor – beta-1 (TGFβ-1), 5ng/mL fibroblast growth factor-2 (FGF-2), and 10ng/mL platelet-derived growth factor-ββ (PDGF-ββ), and changed every two days. Chondrocytes were grown until confluency (6–8 days).

Culture in Alginate

The expanded cells were released from monolayer, resuspended in alginate and cultured as described previously.¹⁶ Alginate beads were depolymerized using a solution of 55mM sodium citrate and 0.15mM NaCl. Chondrocytes and cell associated matrix (CAM) were recovered by centrifugation.

Formation of ARC Constructs

The recovered cells and CAM were resuspended in chondrocyte culture medium at a cell density of 4×10^6 cells/mL. This suspension was used to seed eight 12mm diameter transwell polyester membrane inserts (Corning, Inc., Corning, NY) per donor at 2.5×10^6 cells/cm². Culture medium was changed every other day for 5 weeks.

Transplantation of ARC Constructs into Nude Mice

A total of eight athymic (nu/nu) mice were employed in the *in vivo* study according to a protocol approved by the university's Institutional Animal Care and Use Committee. Four mice were used for each donors' (Donors 1 and 2) constructs. After the mice were anesthetized using an intraperitoneal injection of 100mg/kg ketamine and 10mg/kg xylazine, a 2cm incision was made along the midline of the dorsum of each mouse and two separate subcutaneous pockets were made using blunt dissection. One ARC construct was placed in each of these pockets (same donor) and the incision was closed using 5-0 nylon sutures. Pre- and post-implant constructs were compared with native septal cartilage obtained from Donors 3 and 4.

Termination of In Vivo Culture

Two mice were randomly chosen 30 days post-surgery to be sedated using 100mg/kg ketamine and 10mg/kg xylazine and euthanized by cervical dislocation. The other two mice were euthanized at 60 days post-surgery. An incision over the previous scar was made and the constructs were removed using blunt dissection. Each construct was weighed upon its removal. A 9.6mm diameter disk was punched from the center of each construct for biomechanical testing. The remainder of the construct was divided for structural and biochemical testing. The portions of sample used for biochemical testing were digested, one

with proteinase K (PK) in phosphate-buffered EDTA and the other with pepsin, overnight. The remainder of the construct was used for live/dead assay of viability or placed in optimum cutting temperature (OCT) compound and frozen for histochemical analysis.

Live/Dead Assay

One slice, approximately 0.5-mm thick, was cut from the construct. The slice was stained using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes Inc., Eugene, OR). Calcein-AM and ethidium homodimer-1 dyes were prepared according to package instructions to 100x concentration, and then further diluted by combining 5 μ L of each stain with 500 μ L of phosphate-buffered saline (PBS). The construct slice was added to the dye solution at room temperature and protected from light. After 20 minutes, each slice was rinsed twice in 1mL of PBS (10 minutes per rinse) and again protected from light. After staining, the construct was analyzed for cellular viability using fluorescence microscopy.

Histochemistry

Histological analysis was performed on the constructs to visualize the tissue morphology and to localize GAG. Samples to undergo histochemical analysis were placed in OCT compound and frozen by immersion in liquid nitrogen-cooled isopentane. They were sectioned in a cryostat at either 5 μ m (implanted constructs) or 40 μ m (pre-implant constructs) thickness. For staining with H&E, slides from each sample group were stained sequentially in 0.6% (w/v) Hematoxylin in a solution containing 0.06% (w/v) sodium iodate, 5.28% (w/v) aluminum sulfate hydrate, 25% (v/v) ethylene glycol, and 6% (v/v) glacial acetic acid in water (StatLab Medical Products, McKinney, TX), then Scott's Bluing Reagent (StatLab Medical Products, McKinney, TX), followed by 0.025% (w/v) Eosin Y in a solution containing 54% (v/v) denatured alcohol composed of 5 parts methanol, 3.6% (v/v) isopropanol, 1% (v/v) methyl isobutyl ketone, and 1% (v/v) glacial acetic acid in water (StatLab Medical Products, McKinney, TX), with rinses in water between each stain solution. The histochemical localization of GAG was performed as described in an earlier report.^{9,16}

Quantitative Assay for DNA

The DNA content was determined using portions of the PK digests and the PicoGreen DNA assay as described in a previous report.^{10,16} DNA content was normalized to determine cell number and also to milligram of wet tissue weight.

Quantitative Assay for Glycosaminoglycan (GAG)

The GAG content was determined as reported previously using portions of the PK digests and the dimethyl-methylene blue (DMMB) reaction.¹⁶ GAG content was normalized per milligram of wet tissue weight (before digestion) and to DNA content.¹¹

Quantitative Assays for Type I and II Collagen

The amount of type I and type II collagen in PK digests was quantified by enzyme-linked immunosorbent assay (ELISA) using human type I and native type II collagen kits (Chondrex Inc, Redmond, WA). Collagen was digested by sequential incubation with 5000

U/ml bovine hyaluronidase (Sigma-Aldrich, St. Louis, MO) at 4°C overnight, followed by two incubations with 10mg/mL pepsin (Sigma-Aldrich, St. Louis, MO) dissolved in 0.05M acetic acid at 4°C overnight. Finally, the samples were incubated with 1mg/mL pancreatic elastase (Sigma-Aldrich, St. Louis, MO) at 4°C for 16 hours. Separate type I and type II collagen ELISAs were performed following the manufacturer's instructions. The optical density was read at 490nm using a spectrophotometric plate reader. Collagen values were normalized per mg of weight tissue weight (prior to digestion) and to DNA content.

Mechanical Testing

Following 2 months of *in vivo* implantation, a single construct was tested upon retrieval to assess material properties in tension. A tapered tensile specimen with a gauge region measuring 4mm by 0.8mm was punched through the construct center and then sliced into three layers, each ~0.4 mm thick, using a vibrating microtome. The three layers spanned nearly the full thickness of the construct and comprised a top peripheral layer including the graft surface previously contacting host tissue, a middle layer, and a bottom peripheral layer without the graft surface (outer 50 µm). Tensile tests were performed on a materials testing machine according to methods used previously to test native human septal cartilage.¹² Briefly, specimens were subjected to a tare load of 0.05 N and then elongated to 10% and 20% strain at 0.25%/s with 900 s of relaxation to equilibrium at each strain step. Specimens were then pulled to failure at 5 mm/min. An equilibrium tensile modulus was determined from the slope of a linear least-squares fit of the data at 0%, 10%, and 20% strain steps. A dynamic tensile modulus was determined as the slope of a linear least squares fit of the data between 25–75% of the maximum stress during the pull to failure. Strength and failure strain were determined as the maximum stress and corresponding strain.

Statistical Analysis

Analysis was performed using Systat 10.2 (Systat Software, Chicago, IL). Means are presented ± the standard deviation (SD). Differences in proliferation, DNA, GAG, collagen, and confined compression modulus were assessed using a one-way analysis of variance (ANOVA) to determine the fixed effect of time. If the ANOVA identified an overall significant effect, post-hoc Tukey's HSD tests were used to identify significant differences between time points. A difference was considered significant when $p < 0.05$.

Results

The athymic mice tolerated the neocartilage constructs well. Infection and extrusion were not observed during the course of the study. Upon recovery, each cartilage construct was surrounded by a thin fibrous capsule, separating the neocartilage from the murine tissue. The recovered cartilage constructs resembled normal cartilage with a smooth, firm, and opaque surface of solid, white tissue, which had maintained the original shape of the initial construct. The pre-implant *in vitro*-cultured construct, however, was gelatinous and translucent (Figure 1). A live/dead viability assay performed after recovery of the implanted constructs revealed cell viability of greater than 90% (data not shown).

Histologic examination of the recovered specimens showed neocartilage formation. Pre-implant constructs were soft and fragile, and were sectioned at a thickness of 40 μ m. However, implanted constructs were more robust and were sectioned at a thickness of 5 μ m. H&E staining of the implanted constructs showed homogenous distribution of chondrocytes within a cell matrix, typical of native cartilage.¹³ Alcian Blue staining demonstrated robust staining, indicating the presence of sulfated GAGs. The 60-day implants exhibited architecture more similar to native septal cartilage, in contrary to the 30-day implants (Figure 2).

Biochemical testing demonstrated a significant increase in construct DNA content, a surrogate measure of cell number, from pre-implantation to both 30 and 60-days of *in vivo* culture, and between 30 and 60-day specimens ($p < 0.01$; data not shown). GAG accumulation per construct wet weight decreased significantly after *in vivo* culture at 30 days, and also between 30 and 60-days ($p = 0.015$ and $p < 0.0001$, respectively; Figure 3). Pre-implant construct GAG content was 10.74 ± 0.42 μ g per mg wet weight, while the GAG content in 30-day and 60-day *in vivo* constructs was 8.86 ± 0.38 and 2.73 ± 1.16 μ g per mg wet weight, respectively. Type II collagen content increased significantly in pre-implant constructs (0.02 ± 0.003 μ g per mg wet weight) to 30-day *in vivo* constructs (0.78 ± 0.12 μ g per mg wet weight; $p = 0.004$). Additionally, a significant increase in type II collagen content was seen between 30-day explants and 60-day explants (0.78 ± 0.12 and 1.44 ± 0.006 μ g per mg wet weight, respectively; $p = 0.005$; Figure 4). Type I collagen content was below the limit of detection for the assay (0.08 μ g/mL) for the pre-implant constructs (data not shown). The type I collagen accumulation decreased from 30-day explants to 60 day explants (0.54 ± 0.14 and 0.40 ± 0.10 μ g per mg wet weight, respectively; $p = 0.003$, data not shown). The ratio of type II collagen to GAG content in the pre-implantation constructs was 0.002. This increased to 0.09 after 30 days of *in vivo* culture and 0.53 after 60 days of *in vivo* culture.

The biomechanical testing results were consistent with the biochemical content of the constructs. Only the 60-day explanted constructs possessed sufficient strength to complete accurate tensile testing. The dynamic modulus and failure strain of the 60 day explanted constructs were found to be 4.93 ± 1.49 MPa and 0.34 ± 0.01 mm/mm, respectively. These results are similar to those of native septal cartilage as can be seen in Table 1.

Discussion

In this study, we demonstrated successful *in vivo* implantation and maturation of human septal neocartilage constructs. After both 30 and 60 days of implantation, the macroscopic examination and histological staining revealed the hyaline-like nature of the cartilage specimens. Neocartilage showed typical features of native cartilage.¹³ Cells in the neocartilage appeared round in shape and homogeneously distributed within a cartilage matrix as seen in native cartilage. In addition, Alcian Blue staining of the retrieved constructs after implantation showed a substantial accumulation of GAG. Development and maturation of the neocartilage was also marked by the increasing DNA content, as well as by decreasing levels of GAG and type I collagen with a concurrent increase in type II collagen content.

Maturation of the neocartilage was reflected in the significant gain in mechanical stability between the 30 and 60 day groups. The dynamic modulus and failure strain of the 60 day implanted cartilage was similar to that of native human nasal septal cartilage, while the equilibrium modulus of the 60 day construct was approximately 1/3 that of native septal cartilage. These measures are influenced by different components of the tissue; the equilibrium modulus may be considered a more direct measure of the collagen network integrity, whereas the dynamic modulus is influenced by GAG and the interactions of collagen and GAG. This may suggest that while the biomechanical and biochemical properties of the construct are maturing, they do not yet fully resemble those of native cartilage.

It has been demonstrated that the growth of articular cartilage *in vivo* involves a greater net deposition of collagen than proteoglycan with a concomitant increase in mechanical integrity. In contrast, growth of explanted articular cartilage *in vitro* is associated with a greater net deposition of proteoglycan than collagen with inferior mechanical properties.¹⁴ Based on this we hypothesize that the ratio of type II collagen to GAG may serve as a better indicator of tissue maturity and development than either measure alone. The type II collagen to GAG ratio of native human septal cartilage is 3.03, with a range of 2.82 in young adults to 4.03 in patients over 61 years-old.¹⁵ A previous study by Alexander et al.¹⁶ determined a type II collagen to GAG ratio of 0.03 for human nasal septal chondrocyte constructs matured *in vitro* for 4 weeks. Pre-implantation constructs in this study possessed a type II collagen to GAG content ratio of 0.002 which increased to 0.09 in 30-day explants and 0.53 in 60-day explants. It is evident that with *in vivo* maturation, the ratio of type II collagen to GAG content increased toward levels observed in native tissue.

Our results are consistent with those from other studies in which neocartilage constructs have been implanted into nude mice. Rotter et al.¹⁷ engineered human septal neocartilage using cells of individual patients cultured in autologous medium and from chondrocyte pools cultured in FBS and seeded onto an Ethisorb-polymer. Following implantation into nude mice, the constructs formed from chondrocyte pools showed homogenous distribution of ECM components. Haisch et al.¹⁸ implanted PGLA-polymer human septal cartilage constructs in nude mice for 6 and 12 weeks, and observed synthesis of abundant GAG matrix histologically. They also found that failure load and compressive modulus were not detectably different in 6- and 12-week constructs and native septal cartilage. The superior compressive modulus observed in this study when compared with results from our 60-day implant, may be due to the addition of the polymer. Moreover, work by Farhadi et al.¹⁹ showed that human septal constructs precultured for 2 and 4 weeks demonstrated increased suture retention strength at the time of implantation, and improved tensile and bending stiffness after explantation when compared to freshly seeded chondrocytes.

Human septal chondrocytes embedded in an alginate polymer have also been injected into nude mice. Development of cartilage with this approach, however, is limited by the large pieces of cartilage required to isolate enough cells to achieve a desired density for injection.²⁰ By recovering the chondrocytes and developing neocartilage *in vitro*, we are able to generate a large number of chondrocytes with small amounts of cartilage.

Although the results of this study are encouraging, additional work is needed to produce optimal neocartilage constructs for clinical application. A large animal study is in progress to assess the effect of the defect created during harvesting of the initial piece of native cartilage. In addition, *in vivo* maturation will also be evaluated in the large animal model. In the current study, the neocartilage constructs are 12mm diameter cylinders. Cartilaginous defects of the nose range in size and shape. Further studies will be initiated to produce larger constructs with varying shapes to repair larger defects.

In this study, we show the feasibility of implanting septal neocartilage constructs in athymic mice. The neocartilage constructs exhibit an increase in DNA and type II collagen content with a concomitant decrease in GAG content which is also reflected in the biomechanical properties. The constructs are biocompatible and show potential for *in vivo* maturation, and eventual clinical application.

Acknowledgments

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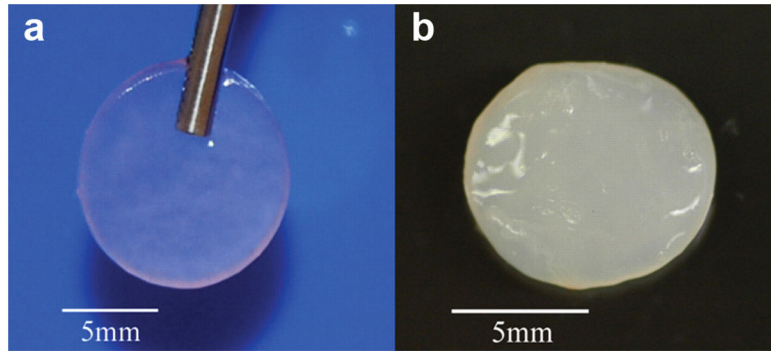


Figure 1. Gross morphologic appearance of pre- (a) and post- (b) 30-day subcutaneous implantation in a nude mouse. The pre-implant construct is gelatinous and translucent. The post-implant construct is firm and opaque, and resembles the gross appearance of native septal cartilage.

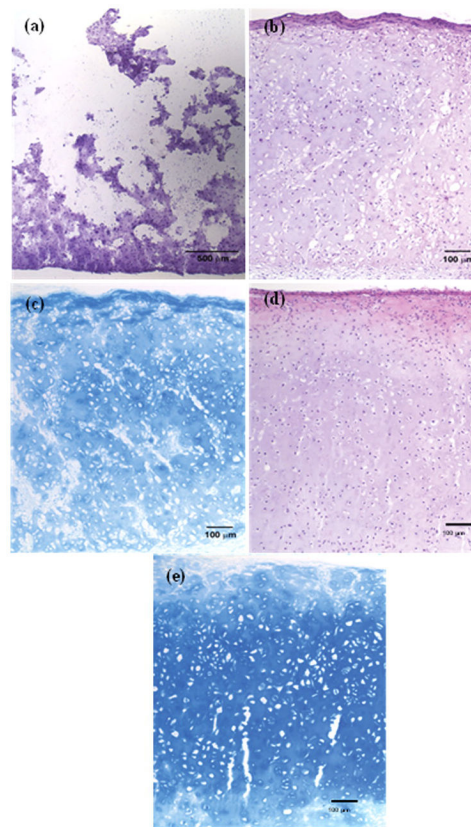


Figure 2. Histological sections stained with H&E and with Alcian Blue for detection of GAG. (a) H&E staining for pre-implant construct sectioned at 40µm. (b) H&E stain of post-30-day implant sectioned at 5µm. (c) Alcian Blue stain of post-30-day implant sectioned at 5µm. (d) H&E stain of post-60-day implant sectioned at 5µm. (e) Alcian Blue stain of post-60-day implant sectioned at 5µm. H&E stain of the neocartilage 30 days post-implantation shows homogeneous distribution of chondrocytes within a cell matrix. At 60 days, the implant exhibits architecture more similar to native septal cartilage.¹³ A moderate to substantial amount of GAG is observed with Alcian Blue staining at 30 and 60 days.

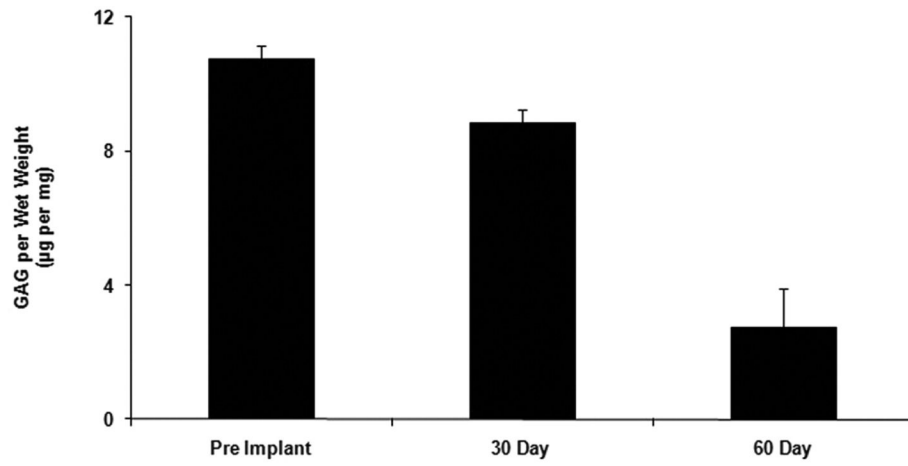


Figure 3. Quantity of GAG per mg of tissue wet weight pre-implantation and after 30 or 60 days of culture *in vivo*. Total GAG content was higher pre-implantation and after 30 days of *in vivo* culture than after 60 days of *in vivo* culture ($p < 0.05$). Error bars depict standard deviation.

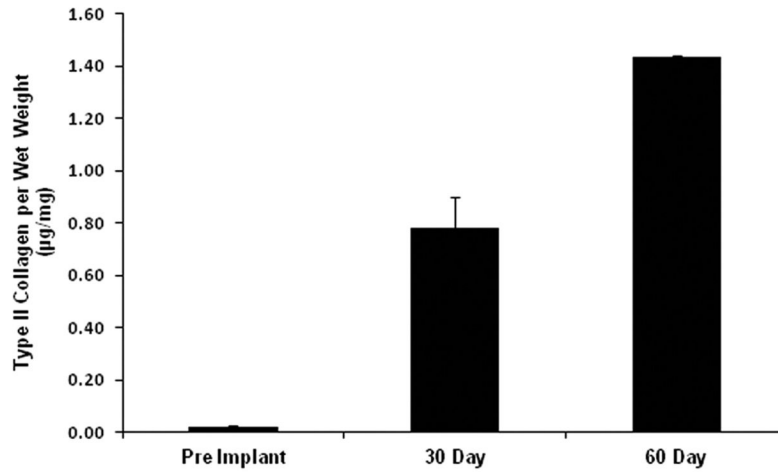


Figure 4.

Quantity of type II collagen per mg of tissue wet weight pre-implantation and after 30 or 60 days of culture *in vivo*. Extractable type II collagen content increased significantly from pre-implantation to 30 days of *in vivo* culture ($p < 0.01$). Type II collagen further increased from 30 days to 60 days of *in vivo* culture ($p < 0.01$). Error bars depict standard deviation.

The mechanical properties of human nasal septal cartilage (huNSC ARC) constructs compared to native human septal cartilage (huNSC Native). Only the 60-day *in vivo* constructs possessed sufficient strength to complete accurate tensile testing. The dynamic modulus and failure strain of the 60-day *in vivo* huNSC ARC constructs (weighted average of 3 construct layers \pm SD) was similar to that of huNSC Native cartilage.

Table 1

Tissue	Equilibrium Modulus (MPa)	Dynamic Modulus (MPa)	Peak Stress (MPa)	Failure Strain (mm/mm)
huNSC ARC Construct				
Preimplant	0.02	-	-	-
30 day <i>in vivo</i>	0.04	-	-	-
60 day <i>in vivo</i>	1.15 \pm 0.35	4.93 \pm 1.49	1.01 \pm 0.16	0.34 \pm 0.01
huNSC Native	3.01	4.99	1.90	0.35